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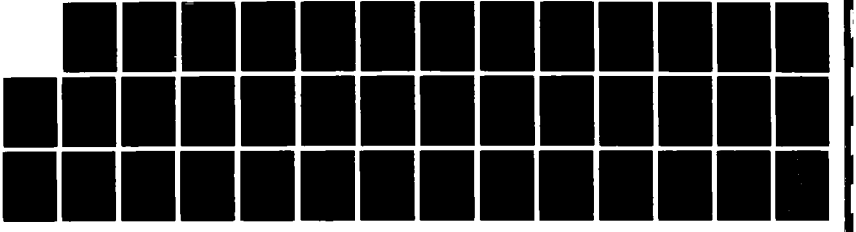

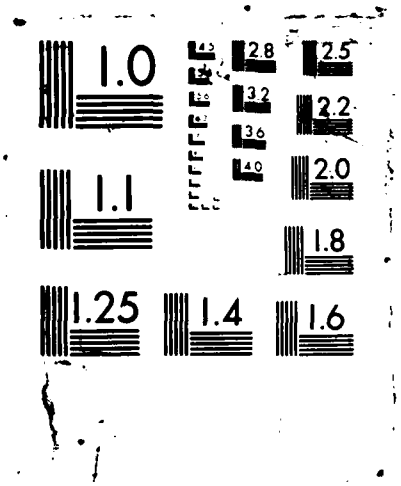
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# ISCHEMIC-ANOXIA OF THE CENTRAL NERVOUS SYSTEM: IRON DEPENDENT OXIDATIVE INJURY DURING REPERFUSION

ANNUAL AND FINAL REPORT

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OCTOBER 15, 1986

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During this period, flunarizine (Ca antagonist) and chloropromazine (effects in myocardium include inhibition of both phospholipase and of lipid peroxidation) for protective effects across the injury parameters were examined. Neither flunarizine nor chloropromazine have so far exhibited marked protective effects.

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## SUMMARY

This work was begun with the recognition that the current practice of resuscitation from cardiac arrest due to either medical or traumatic causes results in major neurologic injury in 50-80% of resuscitated patients.[1,2] Only in the last 25 years has there begun to be a systematic examination of the pathophysiology and molecular biology attendant to brain ischemia and reperfusion. The present work continues to examine the sequence of critical events in brain ischemia and reperfusion and tests therapies which may be applied during the reperfusion phase to inhibit the development of biochemical and functional markers of irreversible brain injury.

The major products of this work are significant advances in the understanding of the pathophysiology of brain ischemia and reperfusion. Our mechanistic hypothesis has developed significantly during our work on this contract, and it may be stated as follows:

1. Complete brain ischemic-anoxia (cardiac arrest) results in rapid loss of tissue high energy charge (also called adenylate charge). The brain adenylate charge is 90% depleted within 5 minutes.[3,4] Degradation of ATP leads to accumulation of adenosine during ischemia,[5] with concomitant accumulation of hypoxanthine.
2. The loss of high energy reserves is immediately followed by loss of normal transmembrane ionic gradients.[6,7] Equilibration between brain intracellular and interstitial concentrations of Na+, K+, and Ca++ has occurred by 10 minutes of complete ischemic-anoxia. The equilibration of the normally very large Ca++ concentration gradient (10,000/1) across the cell membranes is reflected in a 2,000 fold increase in intracellular Ca++ concentration.
3. At least three critical cellular catabolic enzymatic systems are activated by the massive intracellular increases in Ca++. These include
   a. phospholipases, resulting in the liberation of free polyunsaturated fatty acids (PUFAs) from cellular membranes.[8]
   b. an enzyme which proteolytically converts native xanthine dehydrogenase, found in brain capillary endothelium,[9] to xanthine oxidase.[10,11]
   c. endonucleases,[12] which result in the development of single stranded DNA zones and a substantial increase in 5'-$PO_4$ and 3'-OH strand terminals by excission of nucleotides from strands.[13]
4. Brain mitochondrial ability to utilize oxygen to produce ATP by phosphorylation is only moderately impaired by 15 to 45 minutes of complete ischemic anoxia.[14,15] However, these mitochondria do lose 50% of their superoxide dismutase (SOD) activity during the first 15 minutes of complete ischemic anoxia.[14]
5. The situation in the brain at the beginning of reperfusion after a 15 minute cardiac arrest thus includes a zero energy charge; equilibrated transmembrane concentrations of Na+, K+, and Ca+; large increases in intracellular concentrations of adenosine, hypoxanthine, and free PUFAs; xanthine oxidase activity in the capillary endothelium; significant reduction of mitochondrial

superoxide dismutase activity; and the development of areas of single stranded DNA in the nucleus.

6. Initial reperfusion at normal mean cerebral perfusion pressures results in tissue hyperperfusion[16,17] due to profound vasodilatory effects of ATP degradation products[18] accumulated during ischemia. The reintroduction of molecular oxygen by reperfusion results in
   a. Rapid recovery of adenylate charge[3,4] and transmembrane ionic gradients.[4,6]
   b. Oxidative metabolism of hypoxanthine by xanthine oxidase, with concomitant generation of superoxide free radical.[11]
   c. Oxidative metabolism of PUFAs by cyclo-oxygenase and lipoxygenase,[19] with rapid reduction of free PUFA concentrations and concomitant production of prostaglandin and leukotriene products[20] and of superoxide by the cyclo-oxygenase pathway.[19]
7. The brain is ill equipped to handle the superoxide "surge" during reperfusion because of the loss of mitochondrial SOD activity during ischemia[14] and because the nuclei of the selectively vulnerable areas in the cortex, hippocampus, and cerebellum are devoid of the protective enzyme glutathione peroxidase.[21] While superoxide will not directly attack tissue macromolecules,[22] it does reduce ferric iron in storage proteins such as ferritin to soluble ferrous iron.[23] This is reflected in the transition of nearly all brain tissue iron to species weighing less than 30,000 daltons during the first 2 hours of reperfusion[24]. Reductant release of iron in other experimental systems increases as pH falls.[25] This may be critical in severe incomplete ischemia where much deeper tissue acidosis and augmented injury is seen in contrast to complete ischemic-anoxia.[4]
8. The delocalized iron catalyzes the production of powerful radical oxidants either through production of hydroxyl radical (OH*) by iron catalized Fenton chemistry,[26] or by formation of perferryl complexes.[27,28] These species initiate lipid peroxidation chain reactions in cell membranes, which progress over several hours. As these reactions progress and the fatty acid hydroperoxides are preferentialy removed from membrane lipids by phospholipase,[29] lipid peroxidation products appear in the tissue, and selective loss of PUFAs from the brain's lipids becomes evident.[30]
9. The delocalized iron can be bound locally on the sugar-phosphate backbone of DNA.[31] Reactions between this DNA-iron complex and superoxide result in the local formation of OH*[32,33] which attacks the sugar moiety[34,35] resulting in breakage of both sugar-phosphate bonds with loss of the attacked nucleotide[34,35] and a DNA strand break.[32-35] These reactions are rapid and go to completion within about 20 minutes. Occurrance of this reaciton in DNA single stranded zones which developed via endonuclease activity during ischemia results in lethal[36,37] double strand DNA breaks. The selectively vulnerable zones are especially susceptible to this lethal injury becaue their nuclei are devoid of glutathione peroxidase.[21] This vulnerability is reflected in very poor recovery of protein synthesis[3] and dramatic ultrastructural nuclear injury[38] in these zones during reperfusion.

10. The initial phase of tissue hyperperfusion during reperfusion is followed by progressive hypoperfusion.[4,16,39-42] This progressive hypoperfusion is inhibited by treatment during early reperfusion with either Ca++ antagonists[39-42] or the iron chelator deferoxamine and SOD used together.[16] Levels of free arachidonic acid and cyclo-oxygenase products return to normal within the first hour of reperfusion[20]; however, leukotriene levels remain markedly elevated for at least the first 24 hours.[20] 5-HPETE is not only a product of lipoxygenase, but is a fatty acid hydroperoxide readily made by iron dependent peroxidation of arachidonate in situ in membrane lipids. 5-HPETE and its leukotriene derivatives are among the most powerful cerebral vasospastic agents known.[43] These data taken together suggest that production of these leukotrienes is maintained by lipid peroxidation after free arachidonate levels have returned to normal, and that these compounds are responsible for the progressive hypoperfusion phenomenon. Ca++ is the second messenger to the microvascular contractile apparatus.
11. The process of lipid peroxidation during reperfusion ultimately results in loss of membrane integrity.[44] The oxidative processes of lipid peroxidation and DNA double strand scission during reperfusion are both lethal and iron dependent. Both will be subject to therapeutic inhibition by iron chelation. Because lipid peroxidation is fairly slow, relatively large hydrophyllic chelators, such as deferoxamine, which does cross the blood brain barrier slowly,[45] will be effective in inhibiting lipid peroxidation. Such treatment will also change neurologic outcome favorably when cardiac arrest times are short enough that DNA single strand zones and ultrastructural chromatin clumping are not well developed (up to 10 minutes).[46,47] Longer cardiac arrest times will require treatment during reperfusion with small hydrophobic iron chelators such as DETAPAC[48] or methyl-aminopyridones.[49,50] To be protective after prolonged cardiac arrest iron chelators must cross the blood brain barrier very rapidly to prevent the fast and lethal reactions of iron dependent oxidative completion of double strand DNA breaks.

The experiements undertaken under this contract are decribed in detail in the body of this report. We have used a 15 minute cardiac arrest followed by resuscitation with internal cardiac massage in dogs. These experiments have demonstrated tissue iron delocalization to low molecular weight species during the first 2 hours of reperfusion.[24] This has been shown specifically to be a reperfusion phenomenon which does not occur during ischemia.[51] Our experiments have shown that this iron delocalization is accompanied by initiation of lipid peroxidation as demonstrated by measurements of tissue malonidialdehyde, conjugated dienes, and selective loss of PUFAs measured at both 2 and 8 hours of reperfusion.[24,52-54] Both our own experiments and work at Purdue[54] (which has been directly stimulated by theoretical work supported by this contract) demonstrate inhibition of these biochemical markers of lipid peroxidation by deferoxamine. We also examined the time course of tissue ionic content during reperfusion as a functional marker of membrane integrity.[44] Brain tissue ionic content is normal after 2 and 4 hours of

reperfusion; however, by 8 hours of reperfusion, massive tissue Ca++ overloading and reversal of the normal Na+/K+ gradient have occurred. These ionic shifts at 8 hours of reperfusion are also prevented by deferoxamine.[53]

Ultrastructural examination of our dogs' brains after 8 hours of reperfusion was done with support from Michigan State University research funds, and was not supported under this contract. These photomicrographs have been examined both here and in Belgium at the Jansen Research Institute, and they demonstrate profound deterioration of membrane structures in the specificaly vulnerable areas.[55] They also reveal a remarkable loss of nuclear architecture and very extensive chromatin clumping, which is not seen after 15 minutes of ischemia without reperfusion.[38,55] Deferoxamine treated animals showed no evidence of protection from this extensive nuclear injury in the vulnerable areas.[53]

Under this research contract, we conducted a study of the effect of artificial perfusion technique on brain iron delocalization and arterial blood gas parameters.[56] This study demonstrated superior preservation of acid/base stability during open chest cardiac massage (OCCM), in contrast to conventional CPR and interposed abdomenal compression CPR (IAC-CPR). Conventional CPR for 30 minutes following a 15 minute cardiac arrest resulted in significantly greater iron delocalization to LMWS than OCCM or complete ischemia.

We also conducted a prospective, randomized and blind outcome trial using post-resuscitation treatment with deferoxamine and lidoflazine, a calcium antagonist.[57] Neurologic scoring was carried out for 40 hours post-resuscitation, after which the animals were perfusion fixed and their brains examined. No significant differences were found in neurologic deficit scores or in histopathologic scoring of neuronal necrosis in the vulnerable areas. However, while microhemorrhages were widely and consistently found in all areas of standard treatment brains, these lesions were virtually totally absent from the brains of animals treated with deferoxamine and lidoflazine. This finding is very interesting and will be discussed in the body of this report in light of the historic observation of Negovskii[58] that these reperfusion lesions can be prevented by arterial hypoxia.

We thus have strong evidence for reperfusion induced iron delocalization and lipid peroxidation and for the functional results of this process for membrane integrity evaluated by ionic gradients. This, together with the evidence from Purdue[46,47,54] of marked protection of outcome by deferoxamine in a 10 minute cardiac arrest model in rats, strongly indicates that control of tissue iron availability during reperfusion is a critical issue for preserving brain viability. Our inability to demonstrate consistent protection in our outcome study and the photomicrographic evidence of specifically persistent nuclear injury have led us to examine the implications of these reperfusion injury mechanisms for DNA integrity. The results of this literature search are encompassed in the hypothetical model above, will be discussed in the body of this report, and suggest a critical next set of experiments.

If I may be bold at the end of this summary, these experimental and theoretical results have provided a major basis for continued rapid progress in the study of brain injury by ischemic-anoxia, and its prevention. We have enjoyed the financial support and scientific comradeship of the Research and Development Command, and I am proud of both the support and the work which has produced the above results and developments.

## FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an offical Department of Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication Number (NIH) 86-23, Revised 1985).

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## BODY OF REPORT

The Problem:

Closed chest cardiac massage has been the fundamental therapeutic maneuver in resuscitation efforts following cardiac arrest since its introduction in the early 1960s.[59] It was reasoned that provision of some artificially circulated oxygenated blood to vital organs would deter, postpone, or otherwise minimize ischemic injury until normal hemodynamics could be restored. This hope has not been fulfilled by clinical and experimental experience.[1,2,60] This is now generally thought to be due to the inefficiency of CPR in providing brain blood flow. Recent studies of CPR in dogs, pigs, and rabbits using diverse methods for measurement of brain blood flow uniformly demonstrate cortical flow rates of only 3-15% normal.[61-64] This has led to clinical reevaluation of the efficacy of CPR in effecting the outcome of resuscitation. This is currently a controversial area.[65-68] Some groups have been unable to find any effect of bystander CPR on rates of resuscitation or neurologic outcome; however, others have evidence for a very narrow window of protective effect, lasting perhaps no more than 4-6 minutes. This situation leads directly to contemplation of developing therapy to prolong brain toleration to complete ischemia and to reevaluation of more radical and effective artificial perfusion methods suitable only for physician application. These include open chest cardiac massage (OCCM)[60] and emergency cardiac bypass.[69]

Neurologic injury has been the major limiting element in resuscitation. Although the brain is exquisitely sensitive to in-vivo ischemic or anoxic insult, it became apparent from the in-vitro work done by Ames[70,71] that brain neurons have substantial intrinsic resistance to complete ischemic-anoxia. These experiments demonstrated that brain neurons can tolerate between 20 and 30 minutes of complete ischemic-anoxia wihtout losing their ability to recover ATP levels, ionic gradients, action potential generation, and some protein synthetic capability. The situation became more complicated as it was recognized that neurologic injury following ischemic-anoxia and reperfusion was non-uniform. Selectively vulnerable areas in layers 3-5 of the cortex, $CA_1$, and $CA_3$ in the hippocampus, and the purkinje cells of the cerebellum were recognized first histopathologically and then by biochemical markers.[3,4,21,38] These areas have

1. Very poor recovery of protein synthetic capability during reperfusion.[3]
2. The highest specific calcium conductance.[4]
3. Absence of glutathione peroxidase, which is concentrated in the nucleus in other brain cells.[21]

Ultrastructural studies,[38] demonstrated delayed cellular injury which occurs during reperfusion after the initial recovery phase originally demonstrated by Ames. These results are supported directly by our ionic time course experiment (below).

Pieces of the puzzle: Brain perfusion during reperfusion

Immediately following his recognition of evidence of neuronal resistance to ischemic-anoxia, Ames began to look critically at the reperfusion period. Cats were subjected to 20 minutes of brain ischemia by a neck tourniquet model, and the reperfusion pattern was examined by injection of carbon black into their carotid arteries.[72] Non-ischemic controls displayed a uniform pattern of carbon black accumulation in the microvascular macrophages. Examination of post-ischemic animals demonstrated that very little carbon black could be found in the brain. Ames concluded that there was a major defect in microvascular reperfusion, which he labeled "no reflow."

Further study of this situation demonstrated that there was no significant change in intracranial pressure during reperfusion, nor was evidence of occlusive microvascular coagulation found.[73-76] Subsequent quantitative studies of brain reperfusion following ischemic-anoxia by techniques including hydrogen gas clearance, radioactive microspheres, 14C-aminoantipyrine, 133-Xenon, and thermal washout do not, in fact, demonstrate a primary no reflow phenomenon.[17,34-42,73,74,77] The reperfusion pattern at stable perfusion presssures is rather characterized by an initial period of hyperperfusion at rates of 150-200% normal, followed by a progressive development of tissue hypoperfusion which is evident by 20-30 minutes of reperfusion. By 90 minutes of reperfusion following complete ischemic-anoxias of 10-18 minutes, cortical perfusion is found to be at only 15-40% normal. This persists for at least 24 hours.

These findings led to the hypothesis of "secondary ischemia." According to this hypothesis, ischemic injury begun during the primary insult progressed to necrosis during the post-resuscitation hypoperfusion period. Because of the implication of calcium shifts in the induction of ischemic injury (discussed below) and the recognized role of calcium in microvascular myofibrillar contraction, calcium antagonists were investigated for inhibitory effects on the post-resuscitation hypoperfusion syndrome. There is clear evidence from several laboratories that post-resuscitation treatment with flunarizine or nimodipine halts development of post-resuscitation hypoperfusion.[39-42] Contradictory evidence has been developed for the efficacy of lidoflazine in this regard;[78,79] although, the negative study used pretreatment with barbiturate anesthesia and a vascular occlusive model of ischemia which has since been criticized,[80] in contrast to the positive study which used ketamine anesthesia and cardiac arrest. In any event, it is clear that some calcium antagonists will halt the post-resuscitation hypoperfusion phenomenon. However, contrary to the hopes of the investigative teams, improvement in neurologic outcome has been somewhat inconsistent, and a large multicenter clinical study of lidoflazine does not seem to be finding a major effect in patients. Interference with "secondary ischemia" alone does not seem to produce the major neurologic salvage envisioned by this hypothesis. More recently, post-resuscitation treatment with superoxide dismutase and deferoxamine has been shown to inhibit both the initial hyperperfusion phase and the hypoperfusion phenomenon.[16]

Pieces of the puzzle: Calcium

Intracellular calcium ion concentration is normally maintained near 0.1 uM, which is 10,000 fold less than the extracellular Ca++ concentration. This large concentration gradient is maintained by at least four energy dependent pumping systems as follows:[78]

1. A unidirectional pump in the cell membrane which moves Ca++ out of the cell and is directly ATP dependent.
2. A Na+ - Ca++ exchange pump in the cell membrane.
3. A transport system in the mitochondrial membrane which uses the membrane potential established by substrate oxidation and proton pumping to sequester Ca++ in the mitochondrial matrix.
4. A transport system in endoplasmic reticulum which uses ATP to establish a H+ gradient across its membrane, and then uses that gradient to provide the energy for Ca++ uptake.

Complete ischemic anoxia in every major organ system causes rapid decay of the normal Ca++ gradient across the cell membrane. In the brain, equilibration of the extracellular and neuronal Ca++ concentrations occurs and is 80-90% complete during the first 5 minutes of complete ischemic-anoxia.[4,6,7] There are at least 3 Ca++ activated catabolic events which occur during ischemia.

1. Ca++ dependent phospholipase activation[8] leads to deterioration of membrane structures due to hydrolysis of PUFAs from the beta position of lipids. Accumulation of predominantly unsaturated FFAs is well documented during ischemia.[4] If ischemia is prolonged enough (about 1 hour) membrane damage is sufficiently extensive that recovery of ionic gradients during reperfusion can no longer occur.[81]
2. Ca++ dependent activation of a proteolytic enzyme is responsible for the conversion of native xanthine dehydrogenase to xanthine oxidase during ischemia.[10,11] Unlike xanthine dehydrogenase, metabolism of hypoxanthine by xanthine oxidase leads to production of two superoxide anions for each hypoxanthine metabolized.[11] Although there is little xanthine dehydrogenase found in neurons, the enzyme is present in significant quantities in brain capillary endothelium.[9]
3. Ca++ activation of endonucleases[12] is probably responsible for the 150 fold increase in 3'-OH and 5'-$PO_4$ DNA terminals and the development of many single stranded regions in DNA during ischemia.[13] The phenomenon of chromatin clumping which begins to be ultrastructurally evident after about 10 minutes of brain ischemia is well recognized.[38] Endonuclease activation has recently been demonstrated to produce ultrastructurally identical chromatin clumping.[82]

Pre-ischemic treatment with Ca++ antagonists has been uniformly demonstrated to inhibit cellular Ca++ accumulation,[83] FFA accumulation,[8] the development of post-ischemic hypoperfusion,[39,40] structural injury evaluated

by both light and electron microscopic examination, and to remarkably ameliorate ultimate neurologic injury. As noted above and demonstrated in our studies below, administration of calcium antagonists in the post-resuscitation phase has not had such clear protective effects. If ischemia is not so prolonged that membrane integrity has been extensively damaged, Ca++ gradients recover rapidly during early reperfusion. Taken together this evidence indicates that Ca++ may be the major trigger of ischemic injury and set up the conditions for subsequent futher reperfusion injury. However, the evidence does not suggest that Ca++ is itself directly involved in the molecular biology of reperfusion injury.

Pieces of the puzzle: Fatty acid metabolism

Of the FFAs accumulated during ischemia, arachidonic acid metabolism during reperfusion has been extensively studied. More free arachidonic acid accumulates during ischemia than any other FFA,[8] and this fatty acid is the principal precursor for production of the biologically important prostaglandins and leukotrienes.[84] During reperfusion pronounced increases in the tissue concentrations of thromboxane and leukotrienes occur.[20] These compounds are all vasospastic. Unfortunately, the post-ischemic brain chemistry favors production of these compounds more than of vasodilatory prostacyclin.[19,85] Moreover, the circulating half-life of prostacyclin is so short[85] that it is unlikely that significant concentrations of prostacyclin derived from other organs during reperfusion reach the brain's microvasculature. These data suggest that the rapid production of vasospastic prostaglandins and leukotrienes from accumulated arachidonate during reperfusion may represent the primary effectors of the post-resuscitation hypoperfusion phenomenon.

The prostaglandin metabolism of arachidonate via cyclo-oxygenase[21,60] represents another source for excessive production of superoxide during reperfusion.[19] $O_2^-$ is produced as a side product of the oxidative production of PGH.

An important discrepancy exists in the levels of free arachidonate and tissue levels of leukotrienes measured during reperfusion. Free arachidonate levels return to normal within 45 minutes of reperfusion following 15 minutes of brain ischemia.[20] However, elevated leukotriene levels persist even after 24 hours of reperfusion.[20] Since the half-life of leukotrienes is short, the question which immediately occurs (to paraphrase the Wendy's commercial) is where is the substrate? We will suggest below that the persistantly elevated leukotriene levels reflect direct generation of the intermediate hydroperoxide 5-HPETE by lipid peroxidation, and that these products are largely responsible for the prolonged hypoperfusion.

Pieces of the puzzle: Oxygen radicals, iron, and lipid peroxidation

The chemistry of the chain reactions of lipid peroxidation have been studied for the last 40 years with respect to the production of synthetic rubber. Free oxygen radicals were known to be involved in these and other organic oxidation reduction reactions.[27] A free radical is a chemical species with one or more unpaired electrons in an outer orbital. In some organic oxidation reduction (redox) reactions, moleular oxygen ($O_2$) is reduced to $2H_2O$ one electron at a time (univalent reduction).

$$O_2 + e^- \rightarrow O_2^- + e^- \rightarrow H_2O_2 + e^- \rightarrow H_2O + OH^* + e^- \rightarrow H_2O$$

OH* here and below denotes the hydroxyl radical which is not charged. $O_2^-$ denotes the superoxide anion. Both OH* and $O_2^-$ are oxygen radicals. With the 1968 demonstration by McCord and Fridovich[86] that $O_2^-$ was produced by the biochemical reaction between milk xanthine oxidase and hypoxanthine, it became apparent that oxygen radicals might be of significance in biological systems a well as in rubber production. When it was demonstrated that a previously poorly understood plasma protein, erythrocuperin, in fact was a specific catalyst of the dismutation of superoxide to $H_2O_2$,[87] it became certain that these oxygen radical species and their reactions were of major biologic significance. Indeed, oxygen undergoing reduction in mitochondria is reduced univalently, and $O_2^-$ is produced in prostaglandin metabolism and in in-vivo situations where hypoxanthine and xanthine oxidase are present.

Subsequent in-vitro biochemical studies demonstrated that oxygen radicals could attack and degrade major cellular constituents including PUFA's, proteins, and DNA.[26] A flap ensued about the role of $O_2^-$. The Friedovich group believed that this species was itself the direct initiator of damage to cellular constituents. However, the thermodynamicists pointed out, with the certainty of the laws of God, that the Gibbs constant for the reduction of $O_2^-$ (-18 kcal/mole)[88] was not large enough to drive oxidation of the cellular molecules involved. The most readily oxidized cellular species were PUFAs, and the Gibbs constant for loss of the divinyl hydrogen was +58 kcal/mole.[89] In contrast to this situation with $O_2^-$, OH* was known to be a very hot oxidant with a large exothermic Gibbs constant for its reduction to water.[90] This led to examination of reactions in which the presence of $O_2^-$ could lead to the production of OH*. Initially, the Haber-Weiss reaction was proposed

$$2O_2^- \xrightarrow{2H^+} O_2 + H_2O_2$$

$$O_2 + H_2O_2 \longrightarrow O_2 + H_2O + OH^*$$

It was subsequently shown that although this reaction was thermodynamically feasible and the dismutation of $O_2^-$ to $H_2O_2$ occurred at a reasonable rate, in practice the second reaction occurred so slowly that it was called "a non-reaction."[28] Further study of reactions which certainly occurred between oxygen radicals and biological molecules demonstrated that catalysis by transitional metal ions (metals with at least 2 stable ionic valence states) was required.[27,28] Finally, strong evidence for a metal-catalyst-driven Haber-Weiss reaction (also known as the Fenton reaction) was developed. Although iron, copper, and cobalt can catalyse this reaction in-vitro, inadequate amounts of cobalt and inactivation of copper by protein histidine residues leaves iron as the probable biological catalyst.[28]

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$$

$$2O_2^- \xrightarrow{2H^+} H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + Fe^{3+} + OH^*$$

There is also evidence for the formation of active oxidant species in which iron ions of both charges are directly associated with oxygen molecules in perferryl type complexes.[27] It is clear that these complexes themselves may initiate lipid peroxidation without the formation of OH* in some circumstances. It seems likely that this is not an either/or situation biologically, but rather both Fenton chemistry and perferryl type complexes are involved in tissue injury. The critical point is that formation of either type active oxidative species absolutely requires the availability of the metal.[27,28] Probably owing to the insolubility of $Fe^{3+}$ in water ($10^{-18}$ at neutral pH), formation of initiator species and lipid peroxidation occur at increased rates when iron chelators are present which do not completely inactivate the metal to redox participation.[27,28] These include EDTA, ADP, AMP,[27] and DNA.[91] Iron chelators which bind the metal very tightly and which yield inactive metal-chelator complexes include deferoxamine, DETAPAC, o-phenanthroline,[27,28] and alpha-ketohydroxy pyridines.[49] Addition of these compounds to lipid peroxidation systems results in the immediate cessation of the reactions.

In biological membranes as elsewhere, the chain reactions begin with abstraction of a divinyl hydrogen from a PUFA, yielding a reduced oxygen radical and the lipid alkyl radical.[27,92] The lipid alkyl radical then rearranges to a more stable conjugated diene configuration and reacts with $O_2$ to form the lipid peroxyl radical. The lipid peroxyl radical is a potent oxidant and can continue the chain reaction by abstracting the divinyl hydrogen from another PUFA yielding a lipid hydroperoxide and a new lipid alkyl radical. In the presence of a reduced transitional metal, the hydroperoxide group of the peroxidized fatty acid is reductively cleaved, yielding a lipid alkoxyl radical and water.[92] The lipid alkoxyl radical, like the lipid peroxyl radical, is a strong oxidant and can abstract a divinyl hydrogen from an adjacent PUFA, yielding a lipid alcohol and initiating another lipid peroxidation chain.[92] The peroxidized fatty acid remains in the membrane on the original lipid until it is removed by phospholipase, which preferentially cleaves these altered PUFAs.[29,93,94] Once the peroxidized fatty acid has been cleaved off the lipid, it undergoes fragmentation reactions whose products include malondialdehyde, ethane, and pentane.[27,28]

Assays to study lipid peroxidation in in-vivo systems include (see attached manuscript - Krause, GS et al: Natural course of iron delocalization and lipid peroxidation . . . **Ann Emerg Med** in press)

1. spectrophotometric assay for conjugated dienes on isolated lipids
2. spectrophotometric assay of tissue homogenates for the reaction product of MDA with thiobarbituric acid
3. Flouresence spectroscopy of tissue homogenates for the Schiff base formed between MDA and amino groups
4. analysis of expired gases for ethane and pentane
5. conversion of lipid hydroperoxides to MDA followed by reaction with thiobarbituric acid and spectrophotometric assay
6. esterification of isolated lipids followed by quantitative gas chromatography to examine the PUFA content of the total tissue lipids.

There are some problems with all of these assays. For this reason in the studies conducted under this contract we have used three assay methods: MDA, conjugated dienes, and esterification and gas chromotography. We did not use gas evolution because this is a total body assay, and we were explicitly interested in the brain. We did not use lipid hydroperoxide digestion followed by MDA assay because this measurement of lipid hydroperoxides is clearly contaminated by MDA already in the tissue homogenates from in-vivo degradation of lipid hydroperoxides.

Since oxygen radicals are produced in normal mitochondrial and prostaglandin metabolism, it is not surprising that there are cellular defences against the reactions initiated by these species.[26] Vitamin E is an electron donor which is intercalated in biological membranes and serves as a chain terminator for lipid peroxidation. Superoxide dismutase accelerates the conversion of $O_2^-$ to $H_2O_2$, and catalase acts on $H_2O_2$ to produce $2H_2O$ without the intermediate formation of OH*. Glutathione peroxidase catalyses the transition from both $H_2O_2$ and lipid hydroperoxides to their respective alcohols, thereby avoiding formation of OH* or lipid alkoxyl radicals. Unfortunately, the brain, and specifically the cortex is not well equipped to handle oxygen radical species following ischemic insults. The superoxide dismutase activity of mitochondria in the cortex is reduced 50% during 15 minutes of ischemia.[14] The entire brain has only very low concentrations of catalase. Glutathione peroxidase, which is concentrated in the nuclei in other brain cells, is virtually absent in the purkinje cells of the cerebellum, cortex, and hippocampus.[21] Thus, the sum of the evidence reviewed to this point shows that mechanisms to produce excess superoxide upon reperfusion are activated and/or substrate loaded during ischemia, and the homeostatic mechanisms to control oxygen radical reactions are absent or damaged in the areas which display selective vulnerability.

Three central questions remained unaddressed before the work undertaken on this contract. Does iron become available in low-molecular weight species (LMWS) during ischemia or reperfusion? What is the mechanism by which it becomes available? Can iron dependent lipid peroxidation be demonstrated in in-vivo systems? It was known that 10-15% of the total cellular iron was associated with protein species weighing less than 30,000 Daltons,[27] and that concentration of free ionized metal was on the order of $10^{-18}$ M. The normal LMWS iron is thought to be in a "transfer" pool between ferritin storage and use in synthesis of iron containing proteins. Our experiments below demonstrate massive delocalization of tissue iron to the LMWS pool exclusively during reperfusion.[51] Before this result it was quite conceivable that accumulation of NADH during ischemia resulted in transfer of reducing equivalents to FMN and release of $Fe^{2+}$ from ferritin via the classic flavin reduction of $Fe^{3+}$ in ferritin.[95] This is not the case because iron delocalization does not occur even with up to 45 minutes of complete ischemic anoxia.[51] During this contract, other work in our biochemistry laboratories (supported by NIH) demonstrated massive direct release of iron from ferritin by a variety of oxygen radicals behaving as reductants.[96] These include $O_2^-$, adriamycin, and paraquat. We currently believe that the major direct toxicity of $O_2^-$ in biological systems is a result of its properties of both releasing iron from storage molecules and dismuting to $H_2O_2$. Thus superoxide, which will not directly oxidize biological macromolecules, would be directly responsible for the generation of both reactants for in-vivo OH*

generation, and for the availability of iron for formation of perferryl type oxidants.

Pieces of the Puzzle: Protein synthesis, ultrastructure, and DNA

Before we turn to detailed consideration of our experiments executed under this contract, I want to lay the groundwork for the next critical set of experiments which need to be done. This discussion will complete for the Medical Research and Development Command our current understanding of injury mechanisms in ischmia and reperfusion, and provide you with our perception of a next critical scientific step in working out these mechanisms.

There are four lines of indirect evidence implicating nucleic acid injury in the pathophysiology of ischemia and reperfusion. First, the first basic ultrastructural change seen during complete ischemic-anoxia is chromatin clumping, which begins to be evident after 10 minutes of complete ischemia.[38] Second, we have already briefly mentioned the new evidence for single strand DNA injury during ischemia by Ca++ activated endonucleases.[13] Third, when Hossman did regional studies after the initial experiments documenting recovery of new protein synthesis after brain ischemia, he discovered that the vulnerable areas in the cortex had very poor protein synthetic recovery.[3] This did not show significant improvement over 24 hours. At the time, this was attributed to reperfusion injury to ribosomes. Fourth, our ultrastructural studies, conducted in parallel with the demonstration of protection by deferoxamine of PUFAs and membrane function, still demonstrated profound disordering of nuclear architecture in the vulnerable zones.[53]

The single strand breaks which develop during ischemia and are characterized by 5'-$PO_4$ and 3'-OH terminals may be repaired by the DNA polymerase/ligase system against the intact opposite template.[13] However, double strand breaks in DNA are generally thought to be lethal.[36,37] There is some question as to whether double strand breaks may be repaired at all, and there is no biochemical basis for believing that multiple double strand fragments would be reassembled correctly. One DNA double strand break per cell can be lethal.[97] Thus, consideration of DNA injury in ischemia and reperfusion must be concentrated on examination of potential mechanisms by which double strand breaks could occur. In this discussion, we will review in more detail the evidence for endonuclease generated single strand DNA breaks during ischemia and demonstrate available mechanisms by which these might be rapidly converted to lethal double strand breaks during reperfusion.

Warnick et al[13] have recently studied DNA damage during one hour of normothermic ischemia in the kidney. They isolated the DNA from mouse kidneys and used alkaline phosphatase followed by labeling with 32-dATP or 3H-dTTP catalyzed by T4 polynucleotide kinase (specific for 5' terminals) or T4 DNA polymerase (specific for the 3' terminal). These assays were designed and controlled so as to quantitate and characterize the terminals on DNA strand breaks accumulated during ischemia. One hour of warm ischemia causes a 150 fold increase in labeled terminals. The terminals produced by warm ischemia were exclusively 5'-$PO_4$ and 3'-OH in character. Development of single stranded areas was demonstrated by enhanced susceptibility to digestion by S1 nuclease. These specific terminals and the development of single stranded zones support the authors inference that single strand breaks

occur as the result of endonuclease activity during ischemia. Endonucleases are Ca++ activated,[12] and it is not unreasonable to think that they are activated by the massive cellular Ca++ loads which develop during ischemia. Endonuclease activity is also known to cause ultrastructural evidence of chromatin clumping,[82] similar to that seen during ischemia.[38] These findings all suggest that the situation at the initiation of reperfusion includes single stranded zones in the DNA which could be transformed to lethal double strand breaks by only one further biochemical event.

We have already discussed evidence which establishes a strong basis for the assertion that $O_2^-$ is present in excessive concentrations in the brain during the early reperfusion phase following a 15 minute cardiac arrest. Brawn and Fridovich demonstrated single strand DNA scission by enzymatically generated $O_2^-$.[32] This was completely inhibited by 0.1 mM DETAPAC, a high-affinity iron chelator. DETAPAC was shown to have no direct effect on the production of $O_2^-$ by the xanthine oxidase and hypoxanthine system used.[32] Iron was not added to the reactants, and it was concluded that Fe was tightly associated with the purified phage DNA. These investigators suggested that DNA chain scission was caused by generation of hydroxyl radical in the immediate vicinity of the DNA by a Fenton reaction catalyzed by DNA bound iron. Similar experiments by Leski et al[98] also demonstrated that addition of $O_2^-$ to DNA caused strand scission. Addition of $H_2O_2$ to their system caused an 8 fold increase in the velocity of strand scission. Catalase and DETAPAC provided excellent protection.

It is well known that gamma irradiation of aqueous solutions produces OH* by homeolytic cleavage of water; indeed, 70-80% of DNA damage and cell death produced by ionizing radiation is due to OH*.[99,100] Filho and Menghini[33] have studied DNA strand scission in human fibroblasts caused by both $H_2O_2$ and gamma radiation. Addition of $H_2O_2$ to cell cultures resulted in double strand breaks as evaluated by alkaline sucrose density gradient analysis. This effect was maximal at only 28 uM $H_2O_2$. Phenanthroline and bipyridine (both high affinity iron chelators) protected DNA from $H_2O_2$, but did not protect from gamma radiation. Thiourea (100mM), a OH* scavenger, protected DNA from both $H_2O_2$ and Gamma radiation.[33] Titration of the protective concentration of thiourea suggests that OH* travels only 15 to 60 Angstroms from its site of formation before it reacts with DNA. Inhibition of SOD by diethylthiocarbamate (DEC) caused an 8-fold increase in $H_2O_2$ generated strand breaks.[33] DEC was shown not to inhibit catalase, and DEC alone did not cause strand breaks in healthy cells. Phenanthroline caused a 95% inhibition of DNA strand breaks generated by combined treatment with both $H_2O_2$ and DEC.[33] In parallel studies of isolated chromatin, $H_2O_2$ induced DNA fragmentation was inhibited by iron chelators, but it could not be inhibited by extensive dialysis of chromatin.[33] These authors conclude that

1. OH* is the common DNA initiating species for both $H_2O_2$ and gamma radiation.
2. Iron chelators do not inhibit injury by gamma radiation because the production of OH* is a primary consequence of homeolytic cleavage of water. Iron chelators do inhibit $H_2O_2$ induced injury because this injury is mediated by an iron driven Fenton reaction.
3. Iron may be tightly bound to DNA.

4. The large increase in injury seen with inhibition of SOD is due to the transfer of reducing equivalents from $O_2^-$ to iron oxidized to $Fe^{3+}$ by $H_2O_2$, thereby recycling $Fe^{2+}$ for the Fenton reaction.
5. Although OH* has a very short potential diffusion distance, it is formed locally at the DNA by DNA-Fe complex catalyzing the Fenton reaction.

This elegant study thus arrives at the same mechanism as that proposed by Brawn and Fridovich[32] and demonstrates non-dializable binding of iron by native DNA. Shires demonstration of binding of 59Fe to DNA substantiates this evidence.[31] Indeed, the DNA-Fe complex reacts with $H_2O_2$ to form OH* twice as rapidly as occurs in the simple reaction between unbound $Fe^{2+}$ and $H_2O_2$.[91] DNA thus joins the other agents, listed above, which form with ionic iron complexes which are more active in redox catalysis than the uncomplexed metal.

The details of reactions between OH* and DNA are important to the choice of assays to examine the in-vivo significance of these reactions during post-ischemic reperfusion. Dizdaroglu et al[34] subjected DNA in deoxygenated aqueous solutions to OH* attack via gamma irradiation, and carefully analyzed sugars released in the process of strand scission. The results of this analysis led to the suggestion that OH* attacks the DNA deoxyribose moiety at the C-4 carbon, resulting in the initial production of a sugar radical at this position. This is followed by rearrangement of the sugar and elimination of the $PO_4$ from both the 3' and 5' positions of the damaged sugar either by carbocation, or by opening of the ribose ring via water addition followed by beta elimination of both $PO_4$ groups. The result is a missing nucleotide with phosphates remaining on both the 3' and 5' fragment terminals. A similar conclusion regarding the terminals of DNA fragments caused by this process is reached from the electron spin resonance studies of Kuwabara et al.[35] Thus, the appearance of increasing numbers of 3'-$PO_4$ terminals and of DNA fragmentation (which could be demonstrated by agarose gel electrophoresis) during reperfusion would be predicted unique markers of oxygen radical mediated chain scission. The occurance of such a reaction during early reperfusion in the residual single strand following endonuclease injury during ischemia would result in a lethal double strand injury. This possibility is of great concern given

1. the availability of $O_2^-$ during reperfusion,
2. the availability of delocalized LMWS iron during reperfusion,
3. the virtual absence of nuclear protection by glutathione peroxidase and catalase in the selectively vulnerable zones,
4. the fact that DNA damage by this mechanism is completely determined within 20 minutes in in-vitro systems,[101]
5. and the documented supression of protein synthesis and the progression of ultrastructural nuclear injury in the selectively vulnerable zones during reperfusion.[3]

This hypothetical lethal DNA injury fits the available facts and is much more threatening than lipid peroxidation. Lipid peroxidation is a slow process involving bulky hydrophobic compounds densely packed in membranes.

As will be seen in our experiments, the functional consequences of iron dependent lipid peroxidation for ionic gradients do not appear until between 4 and 8 hours of reperfusion. We have lots of lipids to spare. In contrast, it appears that one double strand DNA break per cell may be lethal.[97] In-vivo examination for DNA fragmentation by these mechanisms during reperfusion is the next critical experiment. If this is occurring, use of small hydrophobic iron chelators, possibly administered during a brief period of anoxic reperfusion, may represent a method of inhibiting such determinative damage during early reperfusion and of reaching the holy grail of reliable intact neurologic outcome following prolonged brain ischemic-anoxia.

**OUR EXPERIMENTS**
**9/1/84 - 8/31/86**

The research contract proposal included 4 major experiments using the model of 15 minute cardiac arrest and resuscitation by OCCM and defibrillation:

1. Study of brain tissue at 2 points during the post-resuscitation phase for the molecular weight state of the tissue iron and for markers of lipid peroxidation. This objective was accomplished in three sequential experiments, each conducted with concurrent internal controls.
   A. Brain tissue studies for the concentration of LMWS iron, MDA, conjugated dienes, and the concentration of lipid double bonds in ratio to the concentration of palmitate (Lipid Unsaturation Index: LUSI) were carried out in non-ischemic controls, non-reperfused controls after 15 and 45 minutes of cardiac arrest, and in animals after 2 hours and subsequently after 8 hours of reperfusion.
   B. Based on evidence of iron delocalization and initiation of lipid peroxidation in A above, a time course of tissue ionic concentration gradients was carried out seeking a time displaying unequivocal ionic evidence of membrane dysfunction during reperfusion. Massive shifts toward equilibration of tissue concentrations of Ca++, K+, and Na+ with plasma concentrations of these ions occurred after 4 and by 8 hours of reperfusion. This determined the 8 hour time in I-A.
2. Study of a calcium antagonist and an iron chelator for their effect on LMWS iron and markers of lipid peroxidation. Deferoxamine was studied at both 2 and 8 hours of reperfusion. For obvious reasons based on the above discussion of injury mechanisms, SOD was also studied at 8 hours of reperfusion. Lidoflazine was studied as the Ca++ blocker at 2 hours of reperfusion. During this time, an article appeared challenging the inhibition of the hypoperfusion syndrome by lidoflazine,[79] and we found lidoflazine to be ineffective against markers of lipid peroxidation at 2 hours reperfusion. Because flunarizine was a well characterized Ca++ antagonist and structurally the parent compound to lidoflazine, had been studied extensively in brain ischemia, and incontrovertibly protected from the post-ischemic hypoperfusion syndrome, we elected

to study this compound at the eight hour time. Again, we were able to do concurrent ultrastructural studies on th animals in the 8 hour reperfusion drug studies.

3. Iron delocalization, markers of lipid peroxidation, and arterial oxygenation and acid base balance were studied during 3 methods of artificial perfusion following a 15 minute cardiac arrest. The artificial perfusion methods were conventional CPR, interposed abdominal compression CPR (IAC-CPR), and open chest cardiac massage (OCCM).
4. A prospective, randomized, and blind study was done of combined treatment with lidoflazine and deferoxamine evaluated by neurologic outcome and morphologic examination by light microscopy after 40 hours of reperfusion.

The results of all these experiments have either been published or are submitted for publication in peer reviewed scientific journals. All published abstracts and manuscripts and all submitted manuscripts are included in the Appendices of this report and listed by page number in the Table of Contents. In order that the results of this work may be seen closely assembled, a brief description of the experiements and the tabulated results are presented below. Wherever possible, the data is presented in a time course format. All numeric data is presented as the mean +/- 1 standard deviation unit. Univariant significant differences are inferred at p values less than 0.05. The reader is referred to the manuscripts in the Appendices for the detailed methodology, including detailed description of statistical methodology for each study.

**EXPERIMENT I-A: LMWS Iron and Markers of Lipid Peroxidation**

**TABLE I**

All units are expressed per brain tissue wet weight

| | | **Time Post-Resuscitation** | | |
|---|---|---|---|---|
| **Parameter** | **Non-Isch.** | **15 Min Isch.** | **2 Hours PR** (Post 15 Min Cardiac Arrest) | **8 Hours PR** (Post 15 Min Cardiac Arrest) |
| LMWS Iron nM/100 mg | 9.6 ± 4.9 (n = 11) | 9.3 ± 3.1 (n = 5) | 38.0 ± 4.6 (n = 5) | 2.0 ± .04 (n = 5) |
| MDA nM/100 mg | 7.7 ± 2.0 | 6.1 ± 1.0 | 12.2 ± 1.9 | 40.7 ± 2.3 |
| Conjugated Dienes nM/100 mg | 0.61 ± .11 (n = 6) | 0.59 ± .13 | 1.24 ± .61 | NA |
| LUSI Lipid double bonds/palmitate | 430.2 ± 62.5 (n = 5) | 441 ± 51.3 | 397.2 ± 63.3 | 290.4 ± 61.9 |

Table 1 represents summarized data from 3 manuscripts from the contract work which are attached.

1. Krause, GS, Joyce, KM, Nayini, NR, et al: Cardiac arrest and resuscitation: Brain iron delocalization during reperfusion. **Ann Emerg Med** 1985; 14:1043.
2. Komara, JS, Nayini, NR, Bialick, HA, et al: Brain iron delocalization and lipid peroxidation following cardiac arrest. **Ann Emerg Med** 1986; 15:384-389.
3. Krause, GS, Nayini, NR, Hoehner, TJ, et al: Natural course of iron delocalization and lipid peroxidation during the first 8 hours following a 15 minute cardiac arrest in dogs. Abstract **Ann Emerg Med** 1986; 15:630-631. Full manuscript attached and in press.

There are no significant differences between the data for non-ischemic controls and those for 15 minutes of cardiac arrest without resuscitation. Significant differences ($p < .05$) in the parametric data at 2 and 8 hours of reperfusion are apparent, and statistical analysis is detailed in the attached manuscripts. Conjugated dienes were not done at 8 hours because of tissue sampling limitations. We elected to repeat tissue ions, as detailed below, concurrently in this 8 hour group rather than repeat conjugated dienes here. The large number of non-ischemic controls for basic measurements of iron and MDA reflect our practice of running some concurrent controls in each additional experiment.

These data demonstrate extensive iron delocalization to LMWS exclusively during early reperfusion. After 2 hours of reperfusion, evidence of initiation of lipid peroxidation is apparent in significant increases in MDA and the trend toward increase in conjugated dienes ($p = .06$). At 2 hours of reperfusion, the ratio of lipid double bonds to palmitate (a saturated fatty acid which does not undergo peroxidation) has not changed yet. Although we did not repeat the conjugated dienes measurements at 8 hours reperfusion, Babbs et al[54] have now demonstrated a significant 100% increase in brain conjugated dienes after 4 hours reperfusion following a 10 minute cardiac arrest in rats.

We selected an 8 hour reperfusion time based of tissue ion studies reviewed in I-B below. Our data above at 8 hours reperfusion unequivocally demonstrates iron resequestration from LMWS species by this time; indeed, LMWS iron concentrations are now below non-ischemic levels. Moreover, total tissue iron has not changed during the whole sequence (I-B below). We suggest that the iron delocalization may be a product marker of the reaction of ferritin with excess superoxide formed by metabolism of hypoxanthine and arachidonic acid during about the first hour of reperfusion. Once these substrates are returned to near normal levels, excess superoxide production terminates, and the resequestration of oxidized iron subsequently occurs. However, by this time, lipid peroxidation has already been initiated. The delay in appearance of massively elevated MDA levels and significant loss of PUFAs until 8 hours is not surprising. The appearance of MDA requires not only the peroxidation of the lipid, but also cleavage of the peroxidized fatty acid from the lipid by phospholipase and subsequent molecular degradation. The phospholipase cleavage is the rate limiting step in this

sequence to MDA.[94] Thus, fatty acids which have undergone peroxidation are somewhat delayed in appearing as MDA. Similarly, the fall in LUSI is a measurement of substrate loss rather than product formation; this of course is in a situation in which very large amounts of the lipid substrate are available. Thus, the 30% loss of LUSI by 8 hours of reperfusion in fact documents massive damage to membrane PUFAs. As seen in I-B below, brain tissue LUSI, MDA, and membrane ions are in uniform agreement that massive membrane injury by lipid peroxidation has occurred by 8 hours of reperfusion. These data, the trend in our conjugated dienes at 2 hours, the clear increase in conjugated dienes at 4 hours demonstrated by Babbs et al,[54] and the protective effects of the iron chelator deferoxamine for these parameters (documented in both our studies and those of Babbs) probably represent irrefutable proof of pathophysiologically critical reperfusion injury by iron dependent lipid peroxidation.

**EXPERIMENT I-B: Time Course of Brain Tissue Ion Concentrations During Reperfusion Folloiwng a 15 Minute Cardiac Arrest**

**TABLE II**

Units are per gram of brain tissue wet weight

| | | **Time Post-Resuscitation** | | |
|---|---|---|---|---|
| **Parameter** | **Non-Isch.** (n = 5) | **2 Hours** (n = 5) | **4 Hours** (n = 5) | **8 Hours** (n = 5) |
| Ca uEq/gm | 1.43 ± .15 | 1.45 ± .08 | 1.45 ± .11 | 2.76 ± 1.18 |
| Mg uEq/gm | 5.63 ± .30 | 5.95 ± .46 | 5.61 ± .26 | 4.68 ± 1.14 |
| Fe nEq/gm | 265 ± 54 | 283 ± 29 | 299 ± 35 | 286 ± 42 |
| Na uEq/gm | 60.4 ± 4.9 | 62.1 ± 3.2 | 60.4 ± 4.9 | 107.4 ± 33.2 |
| K uEq/gm | 90.4 ± 10.1 | 96.3 ±10.0 | 75.4 ± 10.2 | 48.5 ± 31.9 |

Table II represents data from the following manuscript produced under this contract.

> Hoehner, TJ, Garritano, AM, DiLorenzo, RA, et al: Brain cortex tissue calcium, magnesium, iron, sodium, and potassium following resuscitation from a 15 minute cardiac arrest in dogs. **Am J Emerg Med** in press for publication January 1987. (galleys attached)

There are no significant differences between non-ischemic controls and post-resuscitation data at 2 and 4 hours of reperfusion. Significant ionic shifts

at 8 hours are apparent. These data demonstrate large shifts of brain tissue ion concentrations, between 4 and 8 hours of reperfusion, toward equilibration with plasma and extracellular fluid concentrations. The shift in Ca concentration is accompanied by reversal of the tissue K/Na gradient. These data suggest a non-calcium dependent development of a major defect in normal membrane ionic impermeability between 4 and 8 hours of reperfusion. This data represents the basis on which we selected 8 hours of reperfusion for the second time at which we studied LMWS iron and markers of lipid peroxidation in Experiment I-A above.

**EXPERIMENT II-A: Effects of Treatment with Deferoxamine and Lidoflazine on LMWS Iron and Markers of Lipid Peroxidation at 2 Hours of Reperfusion**

**TABLE III**

All units are expressed per brain tissue wet weight. Test drugs are given by IV infusion during the first 10 minutes post resuscitation.

| Parameter | Non-Isch | 2 Hours Std. Care | 2 Hours Std. Care and Lidoflazine 1 mg/kg | 2 Hours Std. Care and deferoxamine 50 mg/kg |
|---|---|---|---|---|
| | (n = 11) | (n = 5) | (n = 5) | (n = 5) |
| LMWS Iron nM/100 mg | 9.6 ± 4.9 | 37.0 ± 4.6 | 27.2 ± 3.3 | 24.3 ± 3.4 |
| MDA nM/100 mg | 7.7 ± 2.0 | 12.2 ± 1.9 | 11.5 ± 1.3 | 9.4 ± .08 |
| Conjugated Dienes nM/100 mg | .61 ± .11 | 1.24 ± .61 | 1.21 ± .57 | .64 ± .52 |

Table III represents summarized data from 4 manuscripts from the contract work which are attached.


1. Krause, GS, Joyce, KM, Nayini, NR, et al: Cardiac arrest and resuscitation: Brain iron delocalization during reperfusion. **Ann Emerg Med** 1985; 14:1037-1043.
2. Komara, JS, Nayini, NR, Bialick, HA, et al: Brain iron delocalization and lipid peroxidation following cardiac arrest. **Ann Emerg Med** 1986; 15:384-389.
3. Nayini, NR, White, BC, Aust, SD, et al: Post resuscitation iron delocalization and malondialdehyde production in the brain following prolonged cardiac arrest. **J Free Radicals Biol Med** 1985; 1:111-116.

4. Krause, GS, Nayini, NR, Hoehner, TJ, et al: Natural course of iron delocalization and lipid peroxidation during the first 8 hours following a 15 minute cardiac arrest in dogs. Abstract **Ann Emerg Med** 1986; 15:630-631. Full manuscript attached and in press.

The larger number of non-ischemic controls is the result of our practice of repeating controls concurrently with specific study series. Treatment with lidoflazine had no significant affect on any of the study parameters. Deferoxamine significantly reduced LMWS iron delocalization and accumulation of MDA during the first 2 hours of reperfusion, and the trend towards normalization of conjugated dienes by deferoxamine is evident ($p = .06$). This is confirmed by subsequent studies by Babbs et al[54] demonstrating normalization by deferoxamine of brain conjugated dienes after 4 hours of reperfusion in the 10 minute cardiac arrest model in rats. These data strongly suggest that initiation of lipid peroxidation during the first 2 hours of reperfusion is iron dependent.

The modest but significant reduction of LMWS iron by deferoxamine during the first 2 hours of reperfusion is interesting. There is evidence that the release of iron may itself trigger further iron delocalization,[102] and we have suggested that deferoxamine may have partially inhibited this process.[24] The fact that neither drug prevents substantial iron delocalization may be important for other iron dependent reactions which progress more rapidly than lipid peroxidation, such as radical DNA damage.

**EXPERIMENT II-B: Effects of Treatment with Deferoxamine, Flunarizine, and SOD on LMWS Iron, Lipid Peroxidation, and Tissue Ions at 8 Hours of Reperfusion**

**TABLE IV**

All units are expressed per brain tissue net weight. Test drugs are given by IV infusion during the first 10 minutes post resuscitation.

| Parameter | Non Isch. | 8 Hours Std. Care | 8 Hours Std. Care and Flunarizine 0.1 mg/Kg | 8 Hours Std. Care and SOD | 8 Hours Std. Care and Deferoxamine 200 mg/Kg |
|---|---|---|---|---|---|
| | (n = 5) | (n = 5) | (n = 5) | (n = 5) | (n = 9) |
| LMWS Iron nM/100 mg | 7.9 ±3.9 | 2.0 ±0.4 | 5.8 ±0.9 | 1.6 ±0.6 | 9.1 ±3.2 |
| MDA nM/100 mg | 7.34 ±1.57 | 40.7 ±2.3 | 36.5 ±5.7 | 20.7 ±11.3 | 27.7 ±9.9 |
| LUSI | 430.2 ±62.5 | 290.4 ±61.4 | 295.7 ±55.3 | 268.8 ±35.8 | 439.2 ±48.5 |
| Ca uEq/g | 1.37 ±0.15 | 2.62 ±1.11 | 2.12 ±0.36 | 2.12 ±0.89 | 2.11 ±1.28 |
| Na uEq/g | 54.7 ±6.5 | 95.4 ±27.0 | 71.4 ±11.4 | 64.7 ±5.4 | 64.5 ±14.9 |
| K uEq/g | 81.6 ±11.6 | 41.1 ±15.9 | 65.9 ±18.1 | 59.8 ±7.4 | 76.7 ±15.8 |
| K/Na | 1.36 ±0.1 | 0.48 ±0.24 | 0.94 ±0.34 | 0.93 ±0.11 | 1.22 ±0.27 |

*SOD donated by DDI Pharmaceuticals. Initial dose $10^6$ IU IV followed by 5 x $10^5$ U/hr by IV infusion.

The above study was conducted with all groups being run concurrently for determination of the indicated study parameters. Only this concurrent series of animals are included in the data. A methodologic note regarding the K/Na gradient is in order; the gradient was computed for each individual animal and only then subjected to statistical analysis. All of these animals were also perfusion fixed for pathological examination of the brain by both light and ultrastructural methods. A series of animals was also perfusion fixed after 15 minutes of ischemia without resuscitation; these animals serve as controls in the pathological studies. As noted before, ultrastructural studies were not envisioned in the present contract, nor have they been charged to it. Ultrastructural studies were made possible by a supplementary grant of funds to the Department of Pathology by Michigan State University.

The results of this large study, after discussion with 3 journal editors in chief, have been subdivided in the literature to provide focus on the issues involved with the individual drugs under study. Data from the above study, therefore, is presented in the following attached manuscripts.

1. Krause, GS, Nayini, NR, Hoehner, TJ, et al: Natural course of iron delocalization and lipid peroxidation during the first 8 hours following a 15 minute cardiac arrest in dogs. Abstract **Ann Emerg Med** 1986; 15:630-631. Full manuscript attached and in press.
2. Kumar, K, White, BC, Nayini, NR, et al: Effect of flunarizine therapy on structure and biochemical markers of neuronal injury at 8 hours of reperfusion following a 15 minute cardiac arrest in dogs. In preparation for submission to **Neuropath.**
3. Kumar, K. Goosmann, M, Krause, GS, et al: Ionic and ultrastructural studies in global ischemic dog brain after 8 hours of reperfusion. Submitted to **Acta Neuropath.**
4. Nayini, NR, Kumar, K, March, GG, et al: Effect of superoxide dismutase therapy on biochemical markers and neuronal ultrastructural injury at 8 hours of reperfusion following a 15 minute cardiac arrest in dogs. Submitted to **Am J Emerg Med.**
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As noted in I-A above, the 8 hour standard care data demonstrate resequestration of tissue iron found to be almost all in the LMWS pool after 2 hours of reperfusion. However, the greater than 5-fold increase in MDA, the 30% loss of LUSI, and the inversion of the K/Na gradient demonstrate that lipid peroxidation and membrane injury are extensive. Light microscopic examination of standard care controls reveals numerous interstitial microhemorrhages and extensive development of neurons with pyknotic nuclei and deeply eosinophilic cytoplasm in the selectively vulnerable zones. Ultrastructural examination of these areas reveals nuclear disorganization after 8 hours of reperfusion which is much worse than that seen after 15 minutes of ischemia. Similar observations of reperfusion nuclear deterioration have been made by other investigators.[38] Extensive degeneration of nuclear, cytoplasmic, and endoplasmic cellular membranes is evident except for mitochondrial structure, which appears well preserved.

Both deferoxamine and SOD treatment result in improvement in MDA; which is not significantly different between these two treatments. However, only deferoxamine provides significant protection of LUSI and the K/Na gradient. This probably reflects both the pivotal role of iron in the PUFA damage and the difficulty of getting a 60,000 dalton protein (SOD) through the blood brain barrier so as to deal with the $O_2^-$ derived from intracellular reactions during early reperfusion. Flunarizine treatment does not result in improvement in any of the biochemical parameters.

Qualitative ultrastructural examination of the brains of dogs treated with any of the three test drugs reveals little improvement. This is especially evident in the nuclear disorganization, which does not appear to be ameliorated at all. The pathology department has purchased a quantitative

computerized morphometric analytical system, which arrived about 3 months ago. This system has now been used by Dr. Kumar to examine the light microscopic preparations from the standard care and flunarizine treated groups. This study (manuscript attached) does demonstrate significant protection by flunarizine, but only of neurons in the hippocampus. The development of microhemorrhages, as evaluated by light microscopy, is virtually totally inhibited by deferoxamine (unpublished data). Quantitative morphometry has not been completed on the deferoxamine and SOD treated brains; however, this analysis will be continued at no further direct cost to the contract.

Of the drugs studied, deferoxamine demonstrates a wide spectrum of protection from lipid peroxidation, tissue ionic shifts, and development of microhemorrhages during reperfusion. These data are of critical importance in that they establish the etiologic role of iron mediation in reperfusion injury. Persistant ultrastructural nuclear damage and our inability to demonstrate reliable neurologic outcome protection in a protocol utilizing deferoxamine indicate that this particular drug is no magic bullet. These problems may be due to pharmacologic limitations of deferoxamine related to its penetration into brain tissue. Deferoxamine is present in good concentrations in the normal brain 5 hours after administration.[45] However, direct analysis of the initial rate at which deferoxamine penetrates the non-ischemic blood brain barrier in rats shows it to be quite slow (Bo Hedlund, University of Minnesota, personal communication of data). These investigations have shown that this initial velocity is more than doubled after an ischemic insult to the brain by four vessel oclusion. Unfortunately, this may well be still too slow to allow adequate control of iron mediated DNA damage by oxygen radicals, since these reactions progress much more rapidly than lipid peroxidation. Happily, iron chelators which will inactivate iron catalysis of either lipid peroxidation or DNA injury and are small hydrophobic structures are available.[49,50] The above experiment points the way to their evaluation, to direct biochemical examination of DNA damage during reperfusion, and probably to addition of a DNA analysis to the test parameters for drug intervention.

**EXPERIMENT III: Brain Iron Delocalization During Various Methods of Artificial Perfusion after Prolonged Cardiac Arrest**

This study examined brain LMWS iron, MDA, and arterial blood gases during 30 minutes of resuscitation from a 15 minute cardiac arrest using CPR, IAC-CPR, and OCCM. An attempt was made to model ventilation and administration of $HCO_3^-$ during resuscitation in such a way as to achieve relatively similar $PCO_2$, $PO_2$, and $HCO_3^-$ values among the three techniques during the resuscitations. We were not successful in doing this. Therefore, in addition to the brain tissue data, this study produced another characterization of blood gas differences among the resuscitation techniques, clearly supporting the superior perfusion characteristics of OCCM.[60] The data and their statistical treatment in this study are complex. Therefore, rather than reproduce them here, the reader is referred to the attached full length manuscript.

Significant increases in tissue MDA were not seen with any of the techniques during 30 minutes of artificial perfusion. Tissue LMWS iron was

significantly and most increased by conventional CPR (doubled compared to non-ischemic controls and OCCM). Both closed chest techniques (CPR and IAC-CPR) demonstrated a persistant disturbed V/Q relationship toward hyperventilation, in spite of our adjustment of compression to ventilation ratios downward to 12/1. OCCM did not display V/Q disturbance in either $PO_2$ or $PCO_2$ values at compression to ventilation ratios of 5/1. Both closed chest techniques, after initial correction with administration of $HCO_3^-$, resulted in development of deep metabolic acidosis ($HCO_3^-$ at about 10 meq/L). OCCM maintained good oxygenation, ventilation and acid base balance.

The blood gas differences to some extent confound the demonstration that conventional CPR produced the greatest iron delocalization during resuscitation. This, of course, is what was expected given

1. Very poor brain blood flow rates seen with CPR, especially after prolonged complete ischemia.[60-64]
2. The profound tissue lactic acidosis which occurs in the brain under the influence of such low flow rates.[4]
3. Siesjo's study of the pH dependence of lipid peroxidation in brain tissue homogenates.[103]
4. and Paques' demonstration of accelerated iron release from ferritin in acidotic conditions.[25]

We conclude again from this study that OCCM resuscitation for cardiac arrest is superior to closed chest artificial perfusion techniques in every parameter that we and other investigators have studied. These include[60] cardiac output, myocardial perfusion, brain perfusion, brain mitochondrial stability,[14] acid base stability,[104] V/Q stability,[105] neurologic outcome,[106] and now the initial rate of brain tissue iron delocalization to LMWS. US Army Combat Casualty Management doctrine regarding resuscitation should perhaps take note of this total data pool.

**EXPERIMENT IV: 40 Hour Neurologic Outcome and Histological Evaluation of Lidoflazine and Deferoxamine Therapy for Post-Arrest Encephalopathy**

This study was conducted as a prospective, randomized, blind, placebo controlled experiment. We evaluated the neurologic outcome and histopathologic injury following a 15 minute cardiac arrest and post-resuscitation treatment wih either standard intensive care or standard intensive care plus treatment with lidoflazine and deferoxamine. The study animals were perfusion fixed for pathologic study at 40 hours for 3 reasons.

1. Extensive prior experience with this model in our laboratories indicated that we would have great difficulty keeping the standard care dogs alive beyond about 48 hours. Historically, significant errors in judgement of outcome have been introduced into drug evaluation (thiopental, by another laboratory) by continuing neurologic evaluation after a majority of control animals had died.
2. Once before this prospective trial, and indeed once since (June 1986) we have had uncontrolled animals on the lidoflazine/deferoxamine treatment protocol get up and walk normally by 24 hours post-resuscitation. Thus 40 hours seemed an adequate post-resuscitation evaluation period.

3. Structural evaluation by light microscopy was an important part of this study. We wanted the control and treated brains to be directly comparable in terms of time from resuscitation to fixation. We fixed these brains in-situ by gravity infusion via a left ventricular trochar placed in every case either within 5 minutes of clinical death or while the animals were still alive. The fixation team was kept on site at the University for this procedure in order to absolutely minimize post-mortem histopathologic changes. In practice, this resulted in a maximum of 40 hours being assigned to the interval between resuscitation and fixation.

This study used continuous physician intensive care of the experimental animals in the animal ICU of Michigan State University's College of Veterinary Medicine. The study parameters included neurologic evaluation by repetitive neurologic deficit scoring (NDS), overall performance category scoring (OPC), and extensive histopathologic evaluation of each animals brain, as was detailed in the contract proposal.

**TABLE V**

| **Hours Post Resuscitation** | | **4** | **8** | **16** | **24** |
|---|---|---|---|---|---|
| STANDARD INTENSIVE CARE | Neurologic Deficit Score | 49.0 ± 10.9 | 42.75 ± 12.5 | 34.0 ± 10.0 | 33.2 ± 7.3 |
| | Overall Performance Category | 3.2 ± 1.1 | 2.75 ± 1.0 | 2.4 ± 1.1 | 2.6 ± 1.1 |
| STANDARD INTENSIVE CARE AND LIDOFLAZINE AND DEFEROXAMINE | Neurologic Deficit Score | 47.8 ± 11.6 | 45.4 ± 8.9 | 39.0 ± 12.6 | 33.2 ± 13.3 |
| | Overall Performance Category | 3.6 ± 0.5 | 3.4 ± 0.6 | 3.2 ± 0.8 | 2.8 ± 0.7 |

This study (during the later part of the first contract year) was conducted with optimism following the first pilot protocol animal which achieved apparently intact neurologic outcome. It was planned to have 10 animals in each treatment goup. It was terminated with 5 animals in each treatment group when it became evident that there were no significant differences in NDS, OPC, nor histopathologic evidence of neuronal necrosis. The experimental answer was disappointingly clear, we have not yet found a "magic bullet" treatment which reliably preserves neurons and neurologic

function after prolonged cardiac arrest. The extensive investment of investigator time (significantly reduced by the Army from the original proposal request) and contract moneys were better spent in further investigation of the pathochemical issues bearing on what was happening when. As detailed above, we did not waste that time.

By qualitative histopathologic scoring for necrotic neurons, no significant differences were noted for neuronal necrosis in any brain area in this initial appraisal. These evaluations are now being repeated on the tissue sections generated by this study using quantitative morphometry, as has been described in the 8 hour biochemical studies with flunarizine. This evaluation process will be continued and these data used in the final manuscript for publication of the results of the present study.

One remarkable and very consistent difference was found between the control and treatment groups during the histopathologic studies. Diffuse microhemorrhages are evident in every brain section of the control brains. These microhemorrhages are virtually totally absent from the treated brains. The phenomenon of brain microhemorrhages in the post-ischemic brain was first described by Negovski.[58] His work demonstrates great curiosity about resumption of spontaneous ventilation and restoration of tissue oxygenation following brain ischemia. He was able to demonstrate that hypoxic reperfusion substantially diminished the incidence of post-ischemic microhemorrhages. His work suggests strongly that oxygen species are involved during reperfusion in the development of microhemorrhages. Similar lesions have been shown to be consequent to the in-vivo exposure of either the spinal cord[107] or the brain to small concentrations of $Fe^{2+}$.[108] Negovski's original observations, the brain and spinal cord work, and our evidence are all consistent with a hypothesis of oxygen dependent iron delocalization, followed by free radical induced tissue damage and microhemorrhage.

As one might imagine, we have reviewed time and again the clinical records of the two uncontrolled experimental animals treated with lidoflazine and deferoxamine who achieved apparently intact neurologic outcome. The only thing we can now see about these animals was that in both cases we had difficulty getting them "pink" during the initial resuscitation. In this regard, it is interesting to note again the recent study from Safar's lab.[16] This study documented greatly improved recovery of sensory evoked potentials and prevention of the post-ischemic hypoperfusion syndrome by a treatment protocol including **initial hypoxic reperfusion**, and administration of deferoxamine and SOD at the onset of reperfusion. Taken together, Negovski's observations, our experience, and Safar's recent study suggest that the initial reintroduction of oxygen may be very critical. This would certainly be true if, as suggested by the evidence we reviewed above, rapid lethal double strand DNA breaks were initiated during reperfusion by iron dependent oxygen radical mechanisms.

## FINAL COMMENTS

The current research contract was terminated based on my specific recommendation contained in the second year third quarter interim report. The reasons for that recommendation were basically two fold.

1. Michigan State University's Colleges of Human and Osteopathic Medicine do not have a University Hospital. Rather, clinical efforts at the University are centered in a free standing outpatient clinic with obvious interest in only low acuity illness and HMO arrangements. This facility serves no significant teaching or research functions. Emergency Medicine faculty were compelled to spend 50-90% of their time in a Minor Emergency Clinic in this facility. Additional pay for this service was at a non-competitive hourly rate and was issued only quarterly, after large deductions by the Dean's office for the "faculty practice plan." This situation was ultimately unacceptable to Emergency Medicine as a discipline, and to its faculty. As a direct result, three nationally recognized senior full time faculty members (two were supported co-investigators on this contract) left within 6 months. We have been unable to negotiate out of this trap. Therefore, I have accepted a tenured position at Wayne State University School of Medicine, Division of Emergency Medicine, effective December 1, 1986.
2. This report demonstrates that the basic questions envisioned in the current contract proposal have been experimentally addressed. Further experiments, beyond the scope of this contract, need to be done, and their scope and direction is obvious from this report.

We believe that your research support for our work detailed above has provided a significant advance in the understanding of brain injury attendant to cardiac arrest and resuscitation. Data has been generated which documents major shifts in tissue iron pools, initiation of iron dependent lipid peroxidation during reperfusion, the result of this process for tissue ion homeostasis during the post-resuscitation hours, and potential implications of the choice of artificial perfusion technique for this process. Deferoxamine therapy was extensively investigated, and while the promise of iron chelation therapy was shown, this drug did not cure post-ischemic encephalopathy. Continuous literature study during the contract by our investigative team has opened the way for further experimental work based on our findings. I am deeply grateful for your support and proud of my group and its work. It was a good two years. Thank you.

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